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(54) Title: HIGH THROUGHPUT ASSAYS FOR THE PROTEOLYTIC ACTIVITIES OF CLOSTRIDIAL NEUROTOXINS

(57) Abstract: In this application is described substrates for high-throughput assays of clostridial neurotoxin proteolytic activities. Two types of substrates are described for use in assays for the proteolytic activities of clostridial neurotoxins: (1) modified peptides or proteins that can serve as FRET substrates and (2) modified peptides or proteins that can serve as immobilized substrates. In both types a fluorescent molecule is present in the substrate, eliminating the requirement for the addition of a fluorogenic reagent. The assays described can be readily adapted for use in automated or robotic systems.

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TITLE OF THE INVENTION

High Throughput Assays for the Proteolytic Activities  
of Clostridial Neurotoxins

5        This application claims priority from U.S. Provisional  
application Serial No. 60/235,050 filed on September 25, 2000.

INTRODUCTION

      The clostridial neurotoxins consist of tetanus  
toxin and the seven immunologically distinct serotypes  
10    of botulinum neurotoxin, elaborated by various strains  
of *Clostridium tetani* and *Clostridium botulinum*,  
respectively. They are among the most potent toxins  
known [Simpson, L. L. (1986) Ann. Rev. Pharmacol.  
Toxicol. 26: 427-453; Nieman, D. H. (1991) In:  
15    Sourcebook of Bacterial Protein Toxins (J. Alouf and  
J. Freer, Eds.) pp 303-348, Academic press, New York].  
All references cited herein supra and infra are hereby  
incorporated in their entirety by reference thereto.

      Nonetheless, these toxins have proven to be  
20    highly useful tools for research on the mechanisms of  
neurotransmitter release [Nieman, D. H. (1991) Trends  
Cell Biol. 4: 179-185; Schiavo, et al. (1994) Cell  
Biol. 5: 221-229], and are being used as clinical  
drugs in humans to treat a rapidly expanding group of  
25    muscle dysfunctions including strabismus,  
blepharospasm, cervical dystonia, and hemifacial spasm  
[Jancovic and Brin (1992) New Engl. J. Med. 324: 1186-  
1194; Kessler and Benecke (1997) Neurotoxicology  
18(3): 761-770]. Although accidental botulinum  
30    intoxication is not considered a major public health  
threat, clostridial neurotoxins have long been  
recognized as potential biowarfare or bioterrorist  
agents [Arnon, S. S., et al. (2001) JAMA 285: 1059-  
1070].

The clostridial neurotoxins are synthesized by the bacteria as single-chain proteins of Mr ~ 150,000, which are subsequently cleaved by endogenous proteases to yield a light chain (Mr ~ 50,000) and a heavy chain (Mr ~ 100,000), covalently linked to each other by a disulfide bond [Bandyopadhyay, et al. (1987) J. Biol. Chem. 262: 2660-2663]. The heavy chains contain receptor-binding and translocation domains, required for entry of neurotoxin into target cells. The light chains are zinc metalloproteases, highly specific for certain proteins involved in neurotransmitter release [Montecucco and Schiavo (1994) Mol. Microbiol. 13: 1-8]. Botulinum serotypes A and E cleave the protein SNAP-25, while tetanus toxin and botulinum serotypes B, D, F, and G cleave synaptobrevin (also called VAMP) [Pellizarri, R., et al. (1999) Philos. Trans. Royal Soc. London B. Biol. Sci. 354: 259-268]. Botulinum serotype C cleaves both syntaxin and SNAP-25 [Foran, P., et al. (1996) Biochemistry 35: 2630-2636]. Only one peptide bond is cleaved by each toxin within its substrate, but this is sufficient to inactivate the mechanism of neurotransmitter release. Toxicity is therefore a consequence of clostridial neurotoxin protease activity.

In view of the widespread applications for clostridial neurotoxins in neurological research and in medicine, and because of the possibilities for use as bioweapons, there is an urgent need for highly sensitive and reproducible assays that can be employed to detect the toxins in potentially contaminated food or environmental samples, to accurately quantify the toxins in research reagents or preparations intended for human clinical use, and in the search for anti-toxin drugs. Because botulinum neurotoxins are proteases, it follows that practical assays for this

activity could form the basis for detection, quantification, and drug-screening systems. However, the development of such assays has been hampered by several factors: (1) As noted above, each botulinum neurotoxin will cleave only one peptide bond in a particular protein, raising the possibility that separate assays would be required for each toxin. (2) A considerable body of evidence has been published which indicates that the substrate recognition requirements of clostridial neurotoxin proteases are unusually large, compared to other proteases, and include discontinuous segments of their respective neuronal target proteins. Therefore, one would anticipate that only intact target proteins or very long polypeptides derived therefrom can function as substrates [for review, see Schiavo, G. et al. (1995) In: Clostridial Neurotoxins (C. Montecucco, Ed.) pp 257-274, Springer-Verlag, Berlin]. (3) The clostridial neurotoxin proteases do not hydrolyze short peptides spanning the cleavage sites, and the tertiary structures of the target proteins are critical elements in substrate recognition [Rossetto O. et al. (1994) Nature 372: 415-416; Schiavo, G. et al. (1995) supra; Washbourne, P. et al. (1997) FEBS Lett. 418: 1-5]. (4) Relatively minor changes in substrate structure, such as the replacement of only one amino acid with a similar one, even at some considerable distance from the cleavage site, can result in complete loss of substrate function [Yamasaki, S. et al. (1994) J. Biol. Chem. 269: 12764-12772; Shone and Roberts (1994) Eur. J. Biochem 225: 263-270; Schmidt and Bostian (1997) J. Prot. Chem. 16: 19-26]. Consequently, introduction of non-natural amino acids and/or bulky aromatic or fluorescent groups would be unlikely to result in a functional substrate.

Curently, the most commonly used methods for detecting botulinum toxins in food and for estimating concentrations in preparations for clinical use are the mouse lethality bioassay [Siegel and Metzger (1979) Appl. Environ. Microbiol. 38: 606-611] and the antibody neutralization test [Siegel (1988) J. Clin. Microbiol. 26: 2351-2356]. Both require the use of animals, can take up to four days to complete, and are inherently inaccurate. Furthermore, determination of botulinum toxin concentration with the mouse bioassay cannot be used to predict pharmacological potency [Pearce, L. B. et al. (1997) Toxicon 35: 1373-1412].

Assays have been published which incorporate neurotoxin protease activity as one aspect of the overall method [Ekong, T. et al. (1997) Microbiology 143: 3337-3347; Wictome, M. et al. (1999) Appl. Environ. Microbiol. 65: 3787-3792; Keller, J. et al. (1999) J. Appl. Toxicol. 19: S13-S17]. Nonetheless, they are essentially immunoassays, because quantitation of results requires the production and use of specialized antibodies, capable of distinguishing between cleaved and uncleaved substrate, or between cleavage product and intact substrate. They have been developed only for botulinum serotypes A and B. They require multiple binding, elution, and washing steps, and are impractical for true high-throughput systems.

Other assays for the proteolytic activities of tetanus toxin and of serotypes A and B botulinum toxins have been reported [Shone, C. et al. (1993) Eur. J. Biochem. 217: 965-971; Cornille, F. et al. (1994) Eur. J. Biochem. 222: 173-181; Schmidt and Bostian (1995) J. Prot. Chem. 14: 703-708; Soleilhac, J.-M. et al. (1996) Anal. Biochem. 241: 120-127]. Although these assays could be adapted to high-

throughput formats, they include high pressure liquid chromatography or solid-phase extraction steps, which add significant time, complexity, and expense to the procedures.

5        Recently, an assay for the proteolytic activity of type B botulinum neurotoxin has been published, which uses a fluorescence resonance energy transfer (FRET) substrate and does not require physical separation of products from reactants [Anne, C. et al.  
10        (2001) *Analyt. Biochem.* 291: 253-261]. The publication describes one substrate, suitable for use with botulinum serotype B only. Because of the extreme specificity of each clostridial neurotoxin for a particular peptide bond in a particular substrate, and  
15        the likelihood that structural modifications to any of the substrates will diminish or abolish cleavability (see discussion above), nothing may be inferred from these results with respect to the suitability of similar modifications to the substrates for the other  
20        clostridial neurotoxins, or to modifications of type B substrate other than those described in the publication.

      An assay for the proteolytic activity of type A botulinum toxin has been described (U. S. Patent No.  
25        5,965,699) which can be conveniently used to quantitate, standardize, and compare different preparations of this toxin. The method is readily adapted to high-throughput format, to search for compounds that inhibit botulinum protease activity  
30        (i.e. potential anti-botulinum drugs). However, the assay requires the addition of a fluorogenic reagent (e.g. fluorescamine, but others are known), which reacts with one of the proteolytic products to yield a fluorescent derivative. Furthermore, in some cases,  
35        test samples might contain compounds that react

directly with the fluorogenic reagent to yield fluorescent derivatives, interfering with the measurement of botulinum protease activity.

The assays described in this application are specifically designed to overcome or eliminate all of the difficulties and drawbacks described above. In view of the unusually high degree of substrate specificity and the large substrate recognition requirements exhibited by all clostridial neurotoxins (v.s.), the extensive substrate modifications required for the development of the new assays would appear to render success highly unlikely. Nonetheless, practical assays have been developed, useful for a wide range of applications.

#### SUMMARY

It is one object of this invention to provide substrate peptides suitable for use in fluorescence resonant energy transfer assays (FRET; also known as quenched-signal assays) for the protease activities of clostridial neurotoxins.

It is another object of the present invention to provide substrate peptides suitable for use in solid phase assays for the protease activities of clostridial neurotoxins.

It is another object of this invention to provide methods for the discovery of compounds that inhibit or otherwise modulate clostridial neurotoxin protease activities. Such compounds may be useful in botulinum toxin clinical applications, or as anti-clostridial neurotoxin drugs.

It is another object of this invention to provide methods for determining the concentrations of clostridial neurotoxins in samples (e.g. preparations of toxin intended for human clinical use) or for detecting the presence of BoNTs in food or

environmental samples, based on the proteolytic activities of the toxins and utilizing the substrates described herein.

It is another object of this invention to  
5 provide methods for detecting the presence of clostridial neurotoxins (in food, environmental samples, etc.), based on the proteolytic activities of the toxins and utilizing the substrates described herein.

10 **BRIEF DESCRIPTION OF THE DRAWINGS**

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying drawing.

15 **Figure 1:** Hydrolysis of peptide (1) by recombinant type A catalytic domain.

**Figure 2:** Fluorescence solublized by different concentrations of botulinum toxins, serotypes A, B, D, and F.

20 **DETAILED DESCRIPTION**

The present invention relates to peptides suitable for the determination of clostridial neurotoxin proteolytic activities. The term "clostridial neurotoxins" refers to the seven  
25 serotypes of neurotoxin (types A through G, inclusive) produced by *Clostridium botulinum*, and to tetanus toxin, produced by *Clostridium tetani*.

The invention includes two types of substrates:  
(I), modified peptides or proteins that can serve as  
30 FRET or quenched-signal substrates in assays for the proteolytic activities of clostridial neurotoxins, and  
(II), modified peptides or proteins that can serve as immobilized substrates (i.e. covalently or otherwise bound to a solid phase) in assays for the proteolytic



activities of clostridial neurotoxins. In both types, a fluorescent molecule is present in the substrate, eliminating the requirement for the addition of a fluorogenic reagent. In assays with type (I) substrates, toxin-catalyzed hydrolysis results in a proportional increase in fluorescence. Therefore, physical separation of cleavage products from intact substrate is not necessary. In type (II) assays, separation of products from intact substrate is accomplished by simply transferring all or part of the soluble fraction to another container, followed by quantitation of the fluorescence in the soluble fraction. Circumstances which favor the use of one type of assay over the other are discussed below. The assays are called "high-throughput" because lengthy processing steps such as centrifugation, solid-phase extraction, or chromatography are not needed. Therefore, the assays can be readily adapted for use in automated or robotic systems.

Circumstances favoring the use of one type of assay over the other include: (1), *Cost*. In some instances, synthesis of a type (I) substrate is more expensive than a type (II) substrate for the same serotype. The substrates for botulinum serotype A described in claims (2) and (9) illustrate this situation. The former, a FRET or type (I) substrate, is more costly to produce than the latter. Therefore, if very large numbers of type A assays are anticipated, economics favors the use of assays incorporating substrate (9), or another type (II) substrate. (2), *Available instrumentation*. The most efficient use of type (II) substrates is in multiwell arrays. However, a fluorometer capable of reading such arrays is required. If a multiwell fluorometer is not available, use of a FRET substrate would be indicated.

(3), *Determinations of clostridial protease kinetic constants*. Initial rates of substrate hydrolyses, catalyzed by clostridial neurotoxin protease activities, are most conveniently determined using FRET or type (I) substrates. Measurements of initial rates are required for calculations of kinetic constants, such as  $K_m$ ,  $k_{cat}$  (turnover number); and the binding affinities of inhibitors or other effectors. (4), *Interference of test samples with direct fluorescence measurements*. Properties of certain test compounds, such as quenching, turbidity, or fluorescence, might preclude quantitation of assay results by direct fluorescence measurements. In this situation, use of a solid-phase or type (II) assay is indicated. At the conclusion of the incubation period, samples are removed and the wells are washed to remove all test compounds and enzymes. The amount of uncleaved substrate still bound to each well is then determined by incubation with trypsin, 50-100 micrograms per ml, followed by fluorescence measurements. In this situation, the presence of an inhibitor is indicated by a higher fluorescence reading, compared to the control, instead of a lower reading, as in direct assays. (5) *Test samples bound to solid matrices*. For example, combinatorial chemistry libraries are often attached to resin beads. In this case, use of a FRET substrate is sometimes more convenient than a solid-phase substrate.

Type (I) substrates:

The following are examples of FRET substrates for the proteolytic activities of clostridial neurotoxins. Each contains a fluorescent group (fluorophore) on one side of the cleavage site, and a molecule that quenches that fluorescence on the other side of the cleavage site. Upon neurotoxin-catalyzed hydrolysis,

the fluorophore and quencher diffuse away from each other, and the fluorescence signal increases in proportion to the extent of hydrolysis. Therefore, the occurrence and rate of hydrolysis may be determined by following the increase in fluorescence with a suitable fluorimeter. Addition of fluorogenic reagents, transfer or washing steps, or substrate immobilization are not required.

Sequences of type A botulinum protease substrates described in U. S. Patent No. 5,965,699, all of which can be modified as described below for use as FRET substrates, are herein incorporated by reference.

Substrate (1) (SEQ ID NO:1) is the following peptide:

15

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17  
S N R T R I D X A N Q R A Z R M L

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and "Z" is S-(fluoresceinyl)-cysteine. This peptide is a substrate for the proteolytic activity of type A botulinum neurotoxin. Cleavage occurs between residues 11 (Q) and 12 (R).

Substrate (2) (SEQ ID NO:2) is the following peptide:

25

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17  
S N R T R I D E A N X R A dcC R M L

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and "dcC" is S-(7-dimethylamino-4-methylcoumarin-3-carboxamidomethyl)-cysteine. This peptide is a substrate for the proteolytic activity of type A botulinum neurotoxin. Cleavage occurs between residues 11 (N(epsilon)-(2,4-dinitrophenyl)-lysine) and 12 (R).

35

Substrate (3) (SEQ ID NO:3) is the following peptide:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20  
5 L S E L D D R A D A L Q A X A S Q F E Z  
  
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35  
S A A K L K R K Y W W K N L K

10 Where "X" is N(epsilon)-(2,4-dinitrophenyl)-  
lysine and "Z" is S-(fluoresceinyl)-cysteine. This  
peptide is a substrate for the proteolytic activity of  
type B botulinum neurotoxin. Cleavage occurs between  
residues 17 (Q) and 18(F).

15

Substrate (4) (SEQ ID NO:4) is the following peptide:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20  
L S E L D D R A D A L Q A G A S X F E dcC  
20  
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35  
S A A K L K R K Y W W K N L K

25 Where "X" is N(epsilon)-(2,4-dinitrophenyl)-  
lysine and "dcC" is S-(7-dimethylamino-4-methyl-  
coumarin-3-carboxamidomethyl)-cysteine. This peptide  
is a substrate for the proteolytic activity of type B  
botulinum neurotoxin. Cleavage occurs between residues  
17 (N(epsilon)-(2,4-dinitrophenyl)-lysine) and 18(F).

30

Substrate (5) (SEQ ID NO:5) is the following peptide:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20  
A Q V D E V V D I M R V N V D K V L X R

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38  
 D Q K L Z E L D D R A D A L Q A G A

5 39  
 S

10 Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and "Z" is S-(fluoresceinyl)-cysteine. This peptide is a substrate for the proteolytic activities of types D and F botulinum neurotoxins. Type D cleaves between residues 23 (K) and 24 (L), while type F cleaves residues 22 (Q) and 23 (K).

15 Substrate (6) (SEQ ID NO:6) is the following peptide:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20  
 A Q V D E V V D I M R V N V D K V L E R

20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38  
 D X K L dcC E L D D R A D A L Q A G A

39  
 S

25 Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and "dcC" is S-(7-dimethylamino-4-methylcoumarin-3-carboxamidomethyl)-cysteine. This peptide is a substrate for the proteolytic activities of types D and F botulinum neurotoxins. Type D cleaves between residues 23 (K) and 24 (L). Cleavage by type F occurs  
 30 between residues 22 (N(epsilon)-(2,4-dinitrophenyl)-lysine) and 23 (K).

Substrate (7) (SEQ ID NO:7) is the following peptide:

```
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
5 A Q V D E V V D I M R V N V D K V L E R

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
D X K L mcp E L D D R A D A L Q A G A

10 39
S
```

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and "mcp" is 2-amino-3-(7-methoxycoumarin-4-yl)-propionic acid. This peptide is a substrate for the proteolytic activities of types D and F botulinum neurotoxins. Type D cleaves between residues 23 (K) and 24 (L). Cleavage by type F occurs between residues 22 (N(epsilon)-(2,4-dinitrophenyl)-lysine) and 23 (K).

20 Substrate (8):

Any peptide or protein that can serve as a substrate for the proteolytic activity of any clostridial neurotoxin, said protein or peptide having been modified to contain a signal moiety on one side of the cleavage site, and a moiety on the other side of the cleavage site that quenches or diminishes the magnitude of that signal. When the substrate is cleaved by clostridial neurotoxin proteolytic activity, the two diffuse away from each other and the signal increases in proportion to the amount of cleavage that has occurred. Examples of signal and quench moieties include, respectively: coumarin derivatives and N(epsilon)-(2,4-dinitrophenyl)-lysine; coumarin derivatives and nitrotyrosine; fluorescein

and rhodamine; fluorescein and N(epsilon)-(2,4-dinitrophenyl)-lysine among others known in the art.

The general concept of FRET assays has been known for many proteases. However, knowledge provided by FRET assays for other proteases cannot be applied directly to the development of FRET substrates for clostridial neurotoxin protease activities, due to the extreme substrate specificities, sensitivities to even minor structural changes in substrates, and the very large substrate recognition requirements of the latter enzymes. In view of these complex and stringent limitations, design of FRET substrates for clostridial neurotoxin protease activities, with respect to types of signal and quench moieties and placement within the substrate sequences, is not obvious.

Type (II) substrates claimed:

Peptides described in substrate (9) - (13) are examples of clostridial neurotoxin substrates, intended for immobilization through reaction of the sulfhydryl groups of the C-terminal cysteine residues in the peptides with maleimide groups, the latter covalently bound to the walls of multiwell plates.

Sequences of type A botulinum protease substrates described in U. S. Patent No. 5,965,699, all of which can be modified as described in this application for use as immobilized substrates, are herein incorporated by reference.

Substrate (9) (SEQ ID NO:8) is the following peptide:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20  
fig G G S N R T R I D E A N Q R A T R M L

21 22 23 24  
G G G C

Where flG is N-fluoresceinyl-glycine. This  
5 peptide is a substrate for the proteolytic activity of  
type A botulinum neurotoxin. Cleavage occurs between  
residues 14 (Q) and 15 (R).

Substrate (10) (SEQ ID NO:9) is the following  
10 peptide:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20  
flG G G L S E L D D R A D A L Q A G A S Q  
15 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38  
F E T S A A K L K R K Y W W K N L K  
39 40 41 42  
G G G C  
20

Where flG is N-fluoresceinyl-glycine. This  
peptide is a substrate for the proteolytic activity of  
type B botulinum neurotoxin. Cleavage occurs between  
residues 20 (Q) and 21 (F).

25 Substrate (11) (SEQ ID NO:10) is the following  
peptide:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20  
30 flG G G A Q V D E V V D I M R V N V D K V  
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38  
L E R D Q K L S E L D D R A D A L Q



39 40 41 42 43 44 45 46  
A G A S G G G C

5       Where flG is N-fluoresceinyl-glycine. This  
peptide is a substrate for the proteolytic activities  
of both types D and type F botulinum neurotoxins. With  
type D, cleavage occurs between residues 26 (K) and 27  
(L), while type F catalyzes hydrolysis between  
residues 25 (Q) and 26(K).

10

Substrate (12) (SEQ ID NO:11) is the following  
peptide:

15       1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20  
Z N K L K S S D A Y K K A W G N N Q D G  
  
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38  
V V A S Q P A R V V D E R E Q M A I  
  
20       39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56  
S G G F I R R V T N D A R E N E M D  
  
25       57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74  
E N L E Q V S G I I G N L R H M A L  
  
75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92  
D M G N E I D T Q N R Q I D R I M E  
  
93 94 95 96 97 98 99 100 101 102 103 104 105 106 107  
30       K A D S N K T R I D E A N Q R  
  
108 109 110 111 112 113 114 115 116  
A T K M L G S G C

Where Z is S-fluoresceinyl-cysteine. This peptide is a substrate for the proteolytic activities of types A and E botulinum neurotoxins. Type A cleaves between residues 106 (Q) and 107 (R), while type E catalyzes hydrolysis between residues 89 (R) and 90 (I).

Substrate (13) (SEQ ID NO:12) is the following peptide:

```
10  1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
    Z N K L K S S D A Y K K A W G N N Q D G

    21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
    V V A S Q P A R V V D E R E Q M A I

15  39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
    S G G F I R R V T N D A R E N E M D

    57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74
20  E N L E Q V S G I I G N L R H M A L

    75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92
    D M G N E I D T Q N R Q I D R I M E

25  93 94 95 96 97 98 99 100 101 102 103 104 105 106 107
    K A D S N K T R I D E A N Q A

    108 109 110 111 112 113 114 115 116
30  A T K M L G S G C
```

Where Z is S-fluoresceinyl-cysteine. This peptide is a substrate for the proteolytic activity of type E botulinum neurotoxin. Type E catalyzes hydrolysis between residues 89 (R) and 90 (I). Replacement of arginine-107 (see sequence in substrate (12)) with

alanine prevents cleavage of this substrate by type A botulinum neurotoxin.

Substrate (14):

- 5       Any peptide or protein that can serve as a substrate for the proteolytic activity of any clostridial neurotoxin, said protein or peptide having been modified so that it can be attached on one side of the proteolytic cleavage site to a solid or
- 10 insoluble material. The attachment point can be on either side (i.e. C-terminal or N-terminal) of the cleavage site. Examples of attachment methods include (but are not limited to): (a), reaction of sulfhydryl groups in the substrate peptide molecules with
- 15 maleimide groups pre-attached to the solid material (or, vice versa ); (b), binding of biotin groups in the substrate molecules to avidin or streptavidin groups on the solid material (or, vice versa). Examples of solid materials for use in this context
- 20 include, but are not limited to: (a) multiwell plastic plates; (b), plastic pins or "dipsticks"; (c), agarose beads, silica beads, plastic beads, or other types of spherical or fibrous chromatographic media; (d), nitrocellulose or other types of sheets or membranes.
- 25       On the other side of the cleavage site, opposite from the point of attachment, the substrate contains a moiety that produces a measurable signal, such as, but not limited to, a fluorescent group or a radioactive isotope. When the proteolytic activity of a
- 30 clostridial neurotoxin cleaves such a substrate, the product containing the signal is released into solution. Subsequently, the amount of signal in the soluble fraction is measured. In addition, if necessary, the amount of residual bound signal can

also be measured following solubilization of the latter with a protease such as trypsin.

Immobilized substrate assays have been developed for many types of enzymes, including other proteases. However, the unusually extensive substrate recognition requirements of the clostridial neurotoxins and the relatively large size of the toxin catalytic subunit (i.e. the light chain, Mr ~50,000) argue against the attachment of neurotoxin-specific substrates to solid supports. Such an arrangement would be expected to result in considerable steric hindrance, preventing free access of the toxin to the substrate on all sides. Furthermore, the stringent requirements of clostridial neurotoxins with respect to substrate amino acid sequence indicates that introduction of bulky fluorescent groups or other signal moieties into potential substrates would eliminate functionality. Therefore, application of knowledge gained from earlier immobilized assays for other enzymes to the development of similar assays for clostridial neurotoxin protease activities is not encouraged and is not straightforward.

Abbreviations for the amino acids are:

	A	Alanine
25	D	Aspartic acid
	E	Glutamic acid
	I	Isoleucine
	K	Lysine
	L	Leucine
30	M	Methionine
	N	Asparagine
	Q	Glutamine
	R	Arginine
	S	Serine

T     Threonine  
X     N-epsilon-(2,4 dinitrophenyl) lysine  
Z     S-(fluoresceinyl)-cysteine  
dcC   S-(7-dimethylamino-4-methyl-coumarin-3-  
5     carboxamidomethyl) cysteine  
mcp   2-amino-3-(7-methoxycoumarin-4-yl)-propionic  
      acid  
flG   N-fluoresceinyl-glycine

10       Peptides can be made with commercially available  
automated synthesizers, using reagents and protocols  
obtained from the manufacturers. Amino acids can be  
obtained in chemically-modified ("protected") forms,  
designed so that they will react with the free amino  
15   group of the preceding residue in the peptide chain,  
but not with themselves. Upon completion of synthesis,  
the peptide is cleaved from the resin, protecting  
groups are removed, and the product is purified.  
These preparation protocols and others are well within  
20   the skill of a person in the art.

      In another embodiment, the present invention  
provides a method for screening compounds which alter  
BoNT activity, such as inhibitors of BoNT activity or  
stimulators of BoNT activity. Solutions of BoNT or  
25   recombinant botulinum toxin are incubated with each  
test compound at ambient temperature, transferred to  
solid supports onto which is immobilized a peptide  
substrate for the BoNT enzyme being tested as  
described above, and processed as described above. A  
30   toxin incubated with a test compound which exhibits a  
reduction in the ability of the toxin to cleave the  
peptide substrate relative to unincubated toxin  
indicates a inhibitory compound. Alternatively, a  
toxin incubated with a test compound which exhibits an  
35   increase in the ability of the toxin to cleave the

peptide substrate relative to unincubated toxin indicated a stimulatory compound.

- In another embodiment, the present invention relates to a kit to search for compounds which inhibit or otherwise alter the protease activities of clostridial neurotoxins. Because the biological effects of clostridial neurotoxins are consequences of their protease activities, it follows that compounds which affect these activities might prove useful as anti-toxin drugs or as tools for further toxin research. Examples of compounds that could be tested include combinatorial sets of chemicals, phage display libraries, or arrays of plant extracts. The kit will contain in close confinement, in a box for example:
- optionally, one or more underivatized multiwell plates ("preincubation plates");
  - an equal number of derivatized multiwell substrate plates, which contain substrates for clostridial neurotoxin protease activities bound to the walls of the wells. In most cases, a well will contain substrate for only one clostridial neurotoxin serotype, but combinations of substrates may be used;
  - optionally, an equal number of opaque-wall multiwell plates, suitable for use in a multiwell fluorimeter;
  - one or more of the clostridial neurotoxins or recombinant light chains thereof;
  - optionally dry buffer components.
- Solutions of neurotoxin or light chain in buffer are mixed with test compounds in the same buffer in preincubation plate wells and incubated at ambient temperature for approximately 15 - 30 min. This step allows the compounds to exert effects, if any, on the toxins before exposure to the substrates. As controls,

wells containing toxin or light chain without test compounds are included. Following preincubation, the solutions are transferred to wells in the derivatized plates containing immobilized fluorescent substrate specific for the clostridial neurotoxin being tested. For example, if type A botulinum neurotoxin is tested, then wells could contain the peptide described in substrate (9). Substrate plates are incubated for 1 - 3 hours at 30° - 37°C. During this time, the toxin or light chain protease activity will cleave the immobilized substrate to a certain extent, thereby solubilizing the proteolysis product containing the fluorescent group. Solutions are then transferred to the corresponding wells of opaque-wall plates.

Fluorescence is then quantitated in a multiwell fluorimeter. Wells containing compounds that stimulated or inhibited toxin protease activity will have more or less fluorescence, respectively, compared to control wells containing toxin only.

In another embodiment, the present invention relates to a kit for determining the concentrations of clostridial neurotoxins in samples; for example, it may be used to monitor the various stages of botulinum toxin production, intended for human clinical applications. Use of this kit requires knowledge of which botulinum serotype is present, and the absence of interfering protease(s). If these conditions are not met, use of the third kit described below is indicated. For illustrative purposes, a kit is described for determining concentrations of type A botulinum neurotoxin. The kit will contain in close confinement, in a box for example:

- FRET substrate for type A botulinum neurotoxin, described in substrate (2), dry;

- optionally, dry buffer components;
- optionally, tween-20 detergent;
- type A botulinum neurotoxin standard.

5           A solution of 30 micromolar substrate is prepared in water, buffered at pH 7.3 and containing 0.05% v/v tween-20. Solutions of various known concentrations of type A botulinum neurotoxin are prepared in the same buffer. Toxin is mixed with substrate, the increase in  
10 fluorescence is measured for a period of time, and the initial rate of fluorescence increase is determined from the early (essentially linear) part of the curve. This is repeated for each known concentration of  
15 toxin, establishing a correlation between toxin concentration and rate. Cleavage rates are then determined for samples containing unknown concentrations of type A neurotoxin. By comparison with the standards, the unknown concentrations may be calculated.

20           In another embodiment, the present invention relates to a kit for detecting the presence of clostridial neurotoxins in samples. In addition to detection, the kit will also eliminate interfering proteases that might be present, identify the  
25 serotypes of the neurotoxins, and permit calculations of neurotoxin concentrations. Kits may be used to screen just a few samples, or large numbers of samples at once. The kit will contain in close confinement, in a box for example:

- 30           - a multiwell plate, containing biotinylated antibodies against all serotypes of the clostridial neurotoxins, bound to avidin- or streptavidin- coated wells. The antibodies are specific for the heavy chains of the toxins. Generally, a particular well  
35 would contain antibodies against only one of the



- clostridial neurotoxins, but all would be represented on the plate. However, in cases where volume of test sample is limited, wells could contain more than one type of antibody. The plate is preferably suitable for use in a multiwell fluorimeter;
- optionally, dry buffer components for wash buffer;
  - optionally, dry activation buffer components, containing buffer, dithiothreitol, and zinc chloride;
  - optionally, Tween-20 detergent;
  - Clostridial neurotoxin standards.
  - Type (I) FRET substrates and/or type (II) substrates, the latter preferably immobilized, for example, by attachment to derivatized plastic pins which are commercially available. The pins can be used individually, or may be attached to plate lids in an array that corresponds to that of the plate wells, such that, when a lid is applied to a plate, one pin enters each well.

Solutions of samples suspected of containing clostridial neurotoxins are placed in plate wells. Wells containing antibodies against each clostridial neurotoxin should receive sample, but if sample volume is limited, wells containing multiple anti-neurotoxin antibodies would be used. Plates are incubated for approximately one hour at 30° - 37° C. During this time, clostridial neurotoxins, if present, will bind to the anti-toxin antibodies in the wells. Wells are then washed with wash buffer (typically, 50 mM tris, 0.1% v/v tween-20, pH 7.5, but others may be used) to remove unbound components including, if present, proteases other than the clostridial neurotoxins. Activation solution (20 mM buffer, 10 mM dithiothreitol, 0.50 mM zinc chloride, pH 7.3) is added and incubated at 30° - 37° C for 20 - 30 minutes to activate clostridial neurotoxin protease

activities. Solutions containing FRET substrates are added, corresponding to the type of neurotoxin that would be captured by the antibody in a particular well. After one hour incubation at 30° - 37° C, the amounts of fluorescence in the wells are determined with a fluorimeter. By comparing fluorescence readings in test sample wells to those obtained from control wells (buffers and substrates only) and to readings from wells containing neurotoxin standards, the presence of a clostridial neurotoxin in a sample may be detected, and its concentration determined. By noting the specificity of the antibody in a positive well, the serotype of the clostridial neurotoxin is revealed.

Alternatively, plastic pins derivatized with the appropriate type (II) immobilized substrates are placed in the wells, instead of type (I) FRET substrates. (Circumstances favoring the use of one substrate type over the other are discussed in a preceding section). After incubation, the pins are removed. If the pins were arrayed in a plate lid, corresponding to the well array, this is accomplished simply by removing the lid. The amount of fluorescence in the wells is then determined in a multiwell fluorimeter. Calculations of results are done as discussed above.

In situations where wells containing antibodies against more than one serotype must be used, differentiation among neurotoxin serotypes is enabled by using substrates containing different fluorophores. For example, a well might contain antibodies against botulinum serotypes A, B, E, and F. After incubation of sample, washing, and activation, a multi-substrate plastic pin with a combination of four immobilized type (II) substrates is placed in the well. In this

example, the pin is derivatized with: (1) substrate for botulinum type A described in substrate (9); (2) substrate for botulinum type B described in substrate (10), modified to replace N-fluoresceinyl-glycine with N-rhodaminy-  
5 N-glycine as the N-terminal moiety; (3) substrate for botulinum type E described in substrate (13), modified to replace S-fluoresceinyl-cysteine with N-(7-methoxy-coumarin-4-acetyl)-glycine as the N-terminal moiety; (4) substrate for botulinum type F described in substrate (11), modified to replace N-fluoresceinyl-glycine with S-(4-acetamido-stilbene-2-  
10 2'-disulfonic acid-4'-carboxamidomethyl)-cysteine. Because the four fluorophores have distinct and well-separated emission spectra, the presence of one or more botulinum serotype(s) is/are revealed by  
15 determining the wavelength(s) of any fluorescence remaining in the well after the removal of the plastic pin.

Immobilization of clostridial neurotoxin serotype-specific antibodies may be accomplished in  
20 other ways. For example, instead of substrates, the antibodies are bound to plastic pins or dipsticks, for use either individually or in arrays corresponding to multiwell plates. The pins are immersed in test  
25 samples. If clostridial neurotoxins are present, they will be captured by the antibodies. After rinsing, the pins are immersed in solutions containing activation buffer (see above), followed by addition of type (I) FRET substrates. An increase in fluorescence (compared  
30 to appropriate controls) indicates the presence of neurotoxin. The serotype is revealed by the type of antibody on the pin. The concentration may be calculated from a standard curve constructed with known concentrations of neurotoxin.

Alternatively, plastic pins derivatized with the appropriate type (II) immobilized substrates are placed in the wells, instead of type (I) FRET substrates. (Circumstances favoring the use of one  
5 substrate type over the other are discussed in a preceding section). After incubation (v.s.), the pins are removed. If the pins were arrayed in a plate lid, corresponding to the well array, this is accomplished simply by removing the lid. The amount of fluorescence  
10 in the wells is then determined in a multiwell fluorimeter. Calculations of results are done and conclusions drawn as discussed above.

In another modification, neurotoxin-specific antibodies are bound to the surface of a sheet or  
15 membrane in roughly circular spots, corresponding to a multiwell plate array. The sheet or membrane is immersed in the test sample, followed by rinsing. The sheet is then clamped between the upper and lower halves of a multiwell plate, such that the sheet forms  
20 the bottom of each well. Alternatively, instead of immersing the sheet in the sample, test samples may be added to the wells, then washed out, or removed by drawing through the sheet by application of a vacuum to the lower plate half. Activation buffer and then  
25 type (I) FRET substrates are added to the wells. Results are calculated and conclusions drawn as noted above. Multiwell plates consisting of separable upper and lower halves or chambers, with and without the capability of applying vacuum to the lower chamber,  
30 are commercially available and not specifically claimed in this patent application.

The following examples are illustrative of the practice of the invention but should not be read as limiting the scope thereof. It is understood that  
35 various modifications could be suggested within the

spirit and purview of this application and the scope of the claims.

The following materials and methods were used in the examples below.

5

## Materials and Methods

### Enzyme Preparations

Botulinum toxins were obtained from Food Research Institute, Madison, WI. All preparations appeared to be more than 90% pure, as judged by SDS-PAGE under reducing conditions. Botulinum toxins were used only by immune personnel under Biosafety Level-2 controls, in accordance with the recommendations of the U.S. Centers for Disease Control and Prevention [*Biosafety in Microbiological and Biomedical Laboratories* (1999) U. S. Government Printing Office, Washington, D. C.]. Maleimide-activated 96-well plates were purchased from Pierce Chemical Co., Rockford, IL.

Recombinant botulinum type A light chain was expressed in competent *E. coli* BL21 cells (Stratagene, La Jolla, CA) using a synthetic gene [Ahmed and Smith (2000) *J. Prot. Chem.* **19**: 475-487] and a pET vector based upon the T7 RNA polymerase expression system Studier et al. (1990) *Methods Enzymol.* **185**: 60-89]. Soluble recombinant type A light chain was purified to >90% purity by ion exchange chromatography at room temperature [Li and Singh (1999) *Prot Expr. Purif.* **17**: 339-344]. 12:31 PM

### Peptide Synthesis

The peptide synthesizer was a model 431A from Perkin Elmer-Applied Biosystems, Foster City, CA. We used protocols, reagents, and chemicals obtained from the manufacturer. Rink resin was employed to yield a carboxamide at the C-terminal residues of all peptides. N(alpha)-Fmoc-N(epsilon)-2,4-dinitrophenyl-

lysine and N-FMOC-2-amino-3(7-methoxycoumarin-4-yl)-propionic acid were purchased from Bachem Bioscience, King of Prussia, PA.

Substrates with S-fluoresceinyl cysteine were prepared by reacting the cysteine sulfhydryl group in the substrate with iodoacetamidofluorescein (Pierce Chemical Co., Rockford IL). Similarly, substrates containing S-(7-dimethylamino-4-methyl-coumarin-3-carboxamidomethyl)-cysteine were prepared by reaction of the cysteine sulfhydryl group with N-(7-dimethylamino-4-methylcoumarin-3-yl)iodoacetamide (Molecular Probes, Eugene OR).

Fluorescein was coupled to N-terminal glycine by deblocking the alpha-amino group, followed by reacting the resin-bound protected peptide with 5-carboxyfluorescein-N-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, IL). Alternatively, 5-carboxyfluorescein (Aldrich Chemical Co., Milwaukee, WI.) was loaded into synthesizer cartridges and placed on the instrument as the last residue. Two couplings were required for complete reaction. After deprotection and cleavage from the resins, crude peptides were purified by reverse-phase HPLC.

#### *Preparation of Immobilized-Substrate Assay Plates*

For covalent coupling to maleimide-activated multiwell plates, fluorescent peptides were dissolved at concentrations of 15 to 20  $\mu$ M in 50 mM Tris, 5 mM EDTA, 0.1% tween, pH 7.9. All wells received 100  $\mu$ L of peptide solution, followed by incubation at ambient temperature for 4 - 5 h. Wells were emptied, then incubated in succession (200  $\mu$ L per well, 1 h) with 40 mM 2-mercaptoethanol in Tris-EDTA-tween, then 10 mg/ml BSA in Tris-tween (no EDTA). Finally, wells were washed three times with 200  $\mu$ L of Tris-tween. Assay plates were stored dry at -10° to -20°C.

### *Coupling of Substrates to Derivatized Plastic Pins*

Derivatized pins (also called "gears") can be obtained from Mimotopes, Raleigh NC., with N-FMOC-beta-alanine bound through a spacer moiety to the polymer backbone. After removal of the FMOC with piperidine, the free amino group of the beta-alanine is iodoacetylated by reaction with iodoacetic anhydride. Substrate peptide is then covalently bound by reaction of the sulfhydryl group in the substrate cysteine residue with N-iodoacetyl-beta-alanine on the pins.

### *Assays of Botulinum Toxin Proteolytic Activity*

Botulinum toxins (10-20 µg/ml) were preactivated by incubation at 37°C for 30 min in 20 mM hepes, pH 7.3, 10 mM DTT, 0.50 mM ZnCl<sub>2</sub>, and 0.05% tween. Assays were conducted in 20 mM hepes, 5 mM DTT, 0.25 mM ZnCl<sub>2</sub>, and 0.05% tween, with various concentrations of toxins.

When recombinant type A light chain was employed in the assays, the lyophilized enzyme was reconstituted to 35-50 ug/ml with 40 mM hepes/0.05% tween, pH 7.3, followed by dilution to the desired concentraion in the same buffer. Unlike whole toxin, preactivation of light chain is not necessary. Botulinum toxin or recombinant type A light chain were incubated in the substrate-coated plates for varying times at 35°C in the dark without agitation. Appropriate control wells containing buffer only were included on each plate. Because the maleimide-activated plates have clear walls, they are not optimized for direct fluorescence measurements. Therefore, after incubation, aliquots were transferred to corresponding wells of opaque-wall plates, and fluorescence was measured with a Wallac 1420 multiwell fluorimeter (PerkinElmer Wallac, Gaithersburg MD,

USA). Excitation and emission wavelengths were 485 and 535 nm, respectively, with readings of 1 second per well. All fluorescence values were expressed in arbitrary units as the mean of triplicate

5 determinations. Error bars are standard deviations.

Assays employing FRET substrates were done in cuvettes in a temperature-controlled fluorimeter (Photon Technology International, Newtown, PA). Toxin or light chain was mixed with appropriate FRET

10 substrate, placed in the instrument, and fluorescence was measured for various times.

#### Example 1

Operation of the invention using a type (I) substrate:

15 In this case, the substrate was the peptide described in substrate (1) above, and the enzyme was a recombinant preparation of type A botulinum toxin catalytic domain (also known as "type A light chain"). A solution of 30 micromolar peptide was prepared in  
20 water, buffered at pH 7.3, and containing 0.05% v/v tween-20. Before addition of enzyme, fluorescence was measured to obtain the background or "zero-time" fluorescence. Enzyme was then added to a concentration of two micrograms per ml, and the resulting increase  
25 in fluorescence due to proteolysis of the peptide was measured with time. Assay temperature was 21° C. In the absence of enzyme, fluorescence changed very little with time, less than  $\pm 5\%$ . Results are shown in Fig. 1. The initial rate of hydrolysis can be  
30 calculated from the slope of the line in the early (essentially linear) part of the curve. Using known concentrations of toxin or light chain, a correlation between rate and concentration can be established, allowing calculation of toxin or light chain  
35 concentrations in unknown samples.



### Example 2

Operation of the invention using type (II) substrates:

5        In this example, the substrates were the peptides described in substrate (9) for botulinum type A, substrate (10) for type B, and substrate (11) for types D and F. The "solid material" to which the substrates were immobilized were 96-well microtiter  
10       plates that were chemically modified to contain maleimide groups (see "Materials and methods" section). Figure 2 depicts fluorescence solubilized by different concentrations of botulinum toxins, serotypes A, B, D, and F (3 hours, 35°C). For each  
15       serotype, maximum fluorescence was defined as that solubilized by 1 microgram per ml toxin under these conditions. The Y-axis extends above 100% to accomodate  
12:31 PM the error bars. Panel (a) shows BoNT A and B; panel (b) shows BoNT D and F.

20       It is understood that these descriptions, examples and embodiments are for illustrative purposes only, and that various modifications would be suggested within the spirit and purview of this application and the scope of the appended claims.  
25

What is claimed is:

1. A clostridial neurotoxin substrate comprising  
5 any peptide or protein that can serve as a substrate  
for the proteolytic activity of any clostridial  
neurotoxin, said protein or peptide having been  
modified to contain a signal moiety on one side of the  
cleavage site, and a moiety on the other side of the  
10 cleavage site that quenches the magnitude of that  
signal such that when the substrate is cleaved, an  
increase in signal is produced.

2. The substrate according to claim 1 wherein  
15 said clostridial neurotoxin is botulinum neurotoxin  
serotype A.

3. The substrate according to claim 2 wherein  
said substrate is a peptide identified in SEQ ID NO:1  
20 or SEQ ID NO:2.

4. The substrate according to claim 1 wherein  
said clostridial neurotoxin is botulinum neurotoxin  
serotype B or tetanus toxin.  
25

5. The substrate of claim 4 wherein said  
substrate is identified in SEQ ID NO:3 and SEQ ID  
NO:4.  
30

6. The substrate according to claim 1 wherein  
said clostridial neurotoxin is botulinum neurotoxin  
serotype D or botulinum neurotoxin serotype F.

7. The substrate of to claim 6 wherein said  
35 substrate is chosen from the group consisting of a

peptide identified in SEQ ID NO:5, SEQ ID NO:6, and  
SEQ ID NO:7.

8. A method for detecting the presence of  
5 clostridial neurotoxin proteolytic activity in a  
sample said method comprising  
mixing the sample with a peptide substrate  
according to claim 1, and  
detecting an increase in signal produced from  
10 proteolytic cleavage of said substrate.

9. A method for measuring concentration of  
neurotoxin in a sample, comprising  
mixing the sample with a peptide substrate  
15 according to claim 1,  
measuring an increase in signal with time  
produced from proteolytic cleavage of said substrate  
and,  
determining the concentration of said neurotoxin  
20 by correlation to a standard.

10. The method according to claim 9 wherein said  
neurotoxin is botulinum neurotoxin serotype A.

25 11. The method according to claim 10 wherein said  
peptide substrate is a peptide identified in SEQ ID  
NO:1 or SEQ ID NO:2.

12. The method according to claim 9 wherein said  
30 neurotoxin is botulinum neurotoxin serotype B or  
tetanus toxin.

13. The method according to claim 12 wherein said  
peptide substrate is a peptide identified in SEQ ID  
35 NO:3 or SEQ ID NO:4.

14. The method according to claim 9 wherein said neurotoxin is botulinum neurotoxin serotype D or F.

5        15. The method according to claim 14 wherein said peptide substrate is chosen from the group consisting of a peptide identified in SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7.

10       16. A kit for determining the concentration of a clostridial neurotoxin in a sample, the kit containing in close confinement,

(i) one or more peptide substrates according to claim 1 cleavable by said clostridial neurotoxin;

15       (ii) said clostridial neurotoxin standard.

17. The kit according to claim 16 wherein said clostridial neurotoxin is botulinum neurotoxin serotype A and the peptide substrate is one or both of the peptides identified in SEQ ID NO:1 and SEQ ID NO:2.

18. The kit according to claim 16 wherein said clostridial neurotoxin is botulinum neurotoxin serotype B or tetanus toxin and the peptide substrate is one or both of the peptides identified in SEQ ID NO:3 and SEQ ID NO:4.

19. The kit according to claim 16 wherein said clostridial neurotoxin is botulinum neurotoxin serotype D or F and the peptide substrate is one or more of the peptides identified in SEQ ID NO:5, SEQ ID NO:6, and SEQ ID NO:7.

20. A botulinum neurotoxin substrate comprising  
any peptide or protein that can serve as a substrate  
for the proteolytic activity of any clostridial  
neurotoxin, said protein or peptide having been  
5 modified so that it can be attached on one side of the  
proteolytic cleavage site to a solid material.

21. The substrate according to claim 20 wherein  
said clostridial neurotoxin is botulinum neurotoxin  
10 serotype A.

22. The substrate according to claim 21 wherein  
said substrate is a peptide identified in SEQ ID NO:8  
or SEQ ID NO:11.  
15

23. The substrate according to claim 20 wherein  
said clostridial neurotoxin is botulinum neurotoxin  
serotype B or tetanus toxin.

20 24. The substrate of claim 23 wherein said  
substrate is identified in SEQ ID NO:9.

25 25. The substrate according to claim 20 wherein  
said clostridial neurotoxin is botulinum neurotoxin  
serotype D or serotype F.

26. The substrate of claim 25 wherein said  
substrate is a peptide identified in SEQ ID NO:10.

30 27. The substrate of claim 20 wherein said  
botulinum neurotoxin is botulinum neurotoxin serotype  
E.

28. The substrate of claim 27 wherein said substrate is a peptide identified in SEQ ID NO:11 and 12.

5           29. A method for detecting the presence of clostridial neurotoxin proteolytic activity in a sample said method comprising

          mixing the sample with a peptide substrate according to claim 20, and

10           detecting an increase in signal produced from proteolytic cleavage of said substrate.

          30. A method for measuring concentration of neurotoxin in a sample, comprising

15           mixing the sample with a peptide substrate according to claim 20,

          measuring an increase in signal with time produced from proteolytic cleavage of said substrate and,

20           determining the concentration of said neurotoxin by correlation to a standard.

          31. The method according to claim 30 wherein said neurotoxin is botulinum neurotoxin serotype A.

25

          32. The method according to claim 31 wherein said peptide substrate is a peptide identified in SEQ ID NO:8 or SEQ ID NO:11.

30           33. The method according to claim 30 wherein said neurotoxin is botulinum neurotoxin serotype B or tetanus toxin.

34. The method according to claim 33 wherein said peptide substrate is a peptide identified in SEQ ID NO:9.

5        35. The method according to claim 30 wherein said neurotoxin is botulinum neurotoxin serotype D or F.

36. The method according to claim 35 wherein said peptide substrate is a peptide identified in SEQ ID  
10 NO:10.

37. The method according to claim 30 wherein said neurotoxin is botulinum neurotoxin serotype E.

15        38. The method according to claim 37 wherein said peptide substrate is a peptide identified in SEQ ID NO:11 or SEQ ID NO:12.

39. A kit for determining the concentration of a  
20 clostridial neurotoxin in a sample, the kit containing in close confinement,

- (i) one or more peptide substrates according to claim 20 cleavable by said clostridial neurotoxin;
- (ii) said clostridial neurotoxin standard.

25        40. The kit according to claim 39 wherein said clostridial neurotoxin is botulinum neurotoxin serotype A and the peptide substrate is one or both of the peptides identified in SEQ ID NO:8 and SEQ ID  
30 NO:11.

41. The kit according to claim 39 wherein said clostridial neurotoxin is botulinum neurotoxin serotype B or tetanus toxin and the peptide substrate  
35 is a peptide identified in SEQ ID NO:9.

42. The kit according to claim 39 wherein said clostridial neurotoxin is botulinum neurotoxin serotype D or F and the peptide substrate is a peptide identified in SEQ ID NO:10.

43. The kit according to claim 39 wherein said clostridial neurotoxin is botulinum neurotoxin serotype E and the peptide substrate is one or more of the peptides identified in SEQ ID NO:11 or SEQ ID NO:12.

44. A method for identifying inhibitors or enhancers of proteolytic activity of a clostridial neurotoxin comprising:

- preincubating a neurotoxin with a test compound to make a neurotoxin-compound solution,
- exposing said solution to a substrate of said neurotoxin according to claim 20,
- measuring signal resulting from the proteolysis of said substrate by said neurotoxin, and
- comparing said signal with controls,

wherein an increase in signal indicates a compound which enhances neurotoxin activity and a decrease in signal indicates a compound which inhibits said neurotoxin.

45. The method according to claim 44 wherein said neurotoxin is botulinum neurotoxin serotype A and the substrate is a peptide identified in SEQ ID NO:8 or SEQ ID NO:11.

46. The method according to claim 44 wherein said neurotoxin is botulinum neurotoxin serotype B or



tetanus toxin and the substrate is a peptide identified in SEQ ID NO:9.

47. The method according to claim 44 wherein said  
5 neurotoxin is botulinum neurotoxin serotype D or F and the substrate is a peptide identified in SEQ ID NO:10.

48. The method according to claim 44 wherein said  
neurotoxin is botulinum neurotoxin serotype E and the  
10 substrate is one or more of the peptides identified in SEQ ID NO:11 or SEQ ID NO:12.

49. A method for identifying a serotype of a  
clostridial neurotoxin in a sample suspected of  
15 containing a neurotoxin, the method comprising  
incubating the sample with antibodies against  
each clostridial neurotoxin such that a neurotoxin is  
bound to its serotype-specific antibody,  
removing unbound components,  
20 adding activation solution such that clostridial  
protease is activated,  
adding solutions containing clostridial  
neurotoxin peptide substrates according to claim 1 to  
said activated protease,  
25 detecting signal generated from proteolysis of  
said substrate by said protease, wherein a signal  
above control indicates presence of a neurotoxin, and  
determining the serotype of the clostridial  
neurotoxin by noting the specificity of the antibody.  
30

50. The method of claim 49 wherein the antibodies  
are bound to a solid material.

51. The method of claim 50 wherein the solid  
35 material is a multiwell plate.

52. The method of claim 51 wherein each well contains an antibody specific for a different neurotoxin serotype.

5

53. The method of claim 49 wherein each peptide substrate is labeled with a different signal.

54. A kit for identifying a serotype of a  
10 clostridial neurotoxin in a sample suspected of  
containing a neurotoxin, comprising  
serotype-specific antibodies  
clostridial neurotoxin standards, and  
peptide substrates according to claim 1.

15

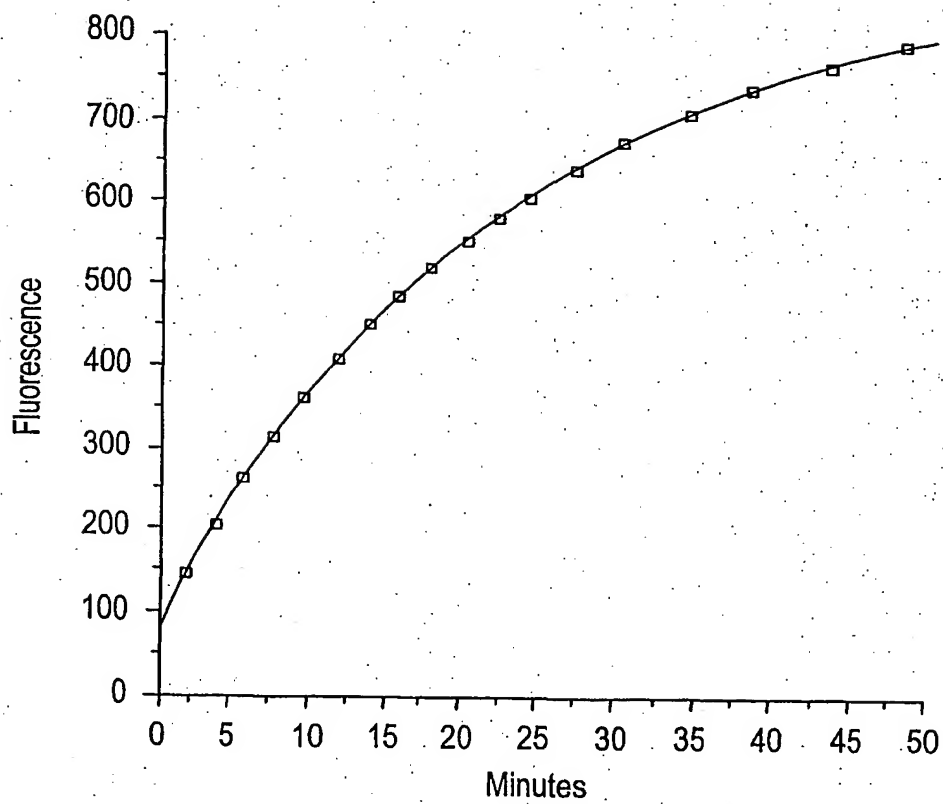
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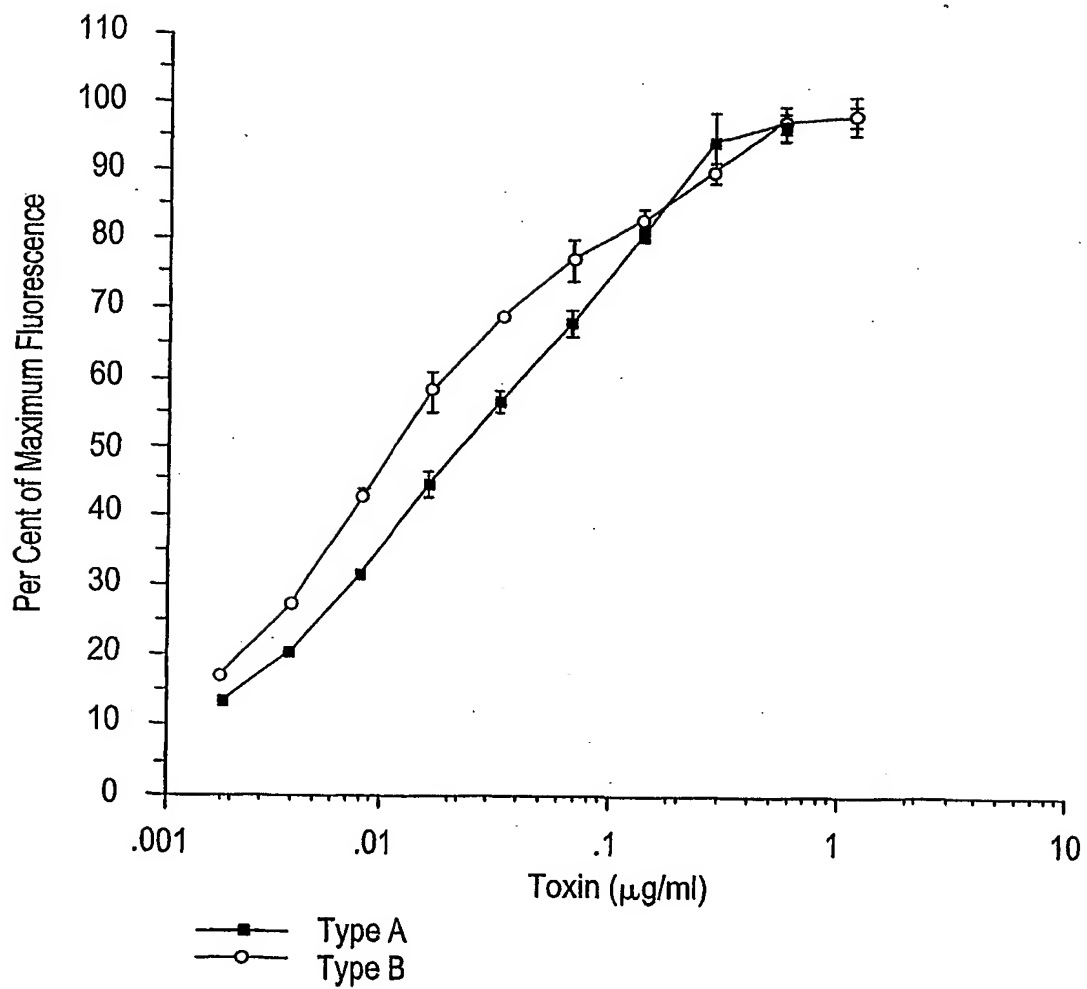
1/3

FIG. 1



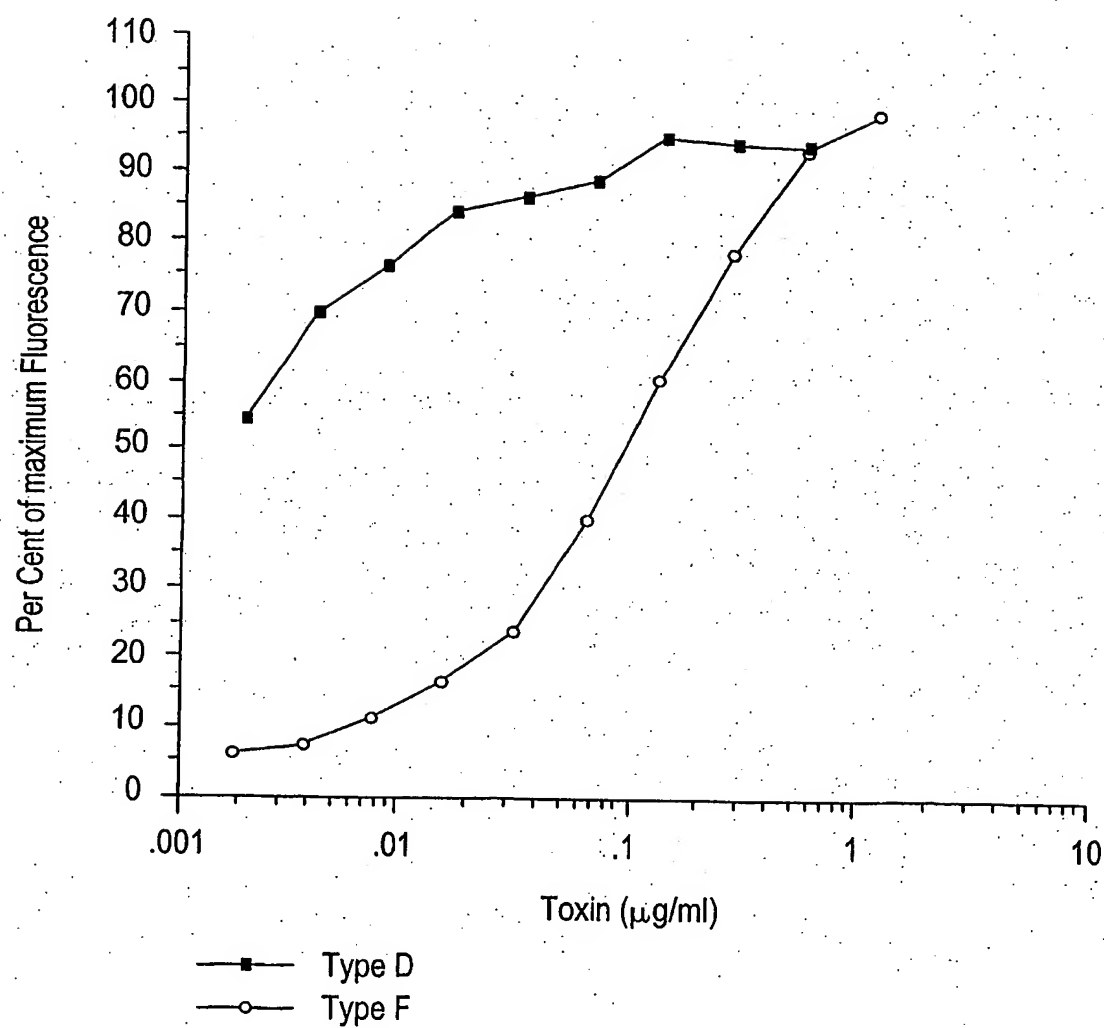
2/3

FIG. 2a



3/3

FIG. 2b



## Sequence Listing

## 1. SEQ ID NO:1

S N R T R I D X A N Q R A Z R M L

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and  
"Z" is S-(fluoresceinyl)-cysteine.

## 2. SEQ ID NO:2

S N R T R I D E A N X R A dcC R M L

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and  
"dcC" is S-(7-dimethylamino-4-methyl-coumarin-3-  
carboxamidomethyl)-cysteine.

## SEQ ID NO:3

L S E L D D R A D A L Q A X A S Q F E Z  
S A A K L K R K Y W W K N L K

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and  
"Z" is S-(fluoresceinyl)-cysteine.

## SEQ ID NO:4

L S E L D D R A D A L Q A G A S X F E dcC  
S A A K L K R K Y W W K N L K

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and  
"dcC" is S-(7-dimethylamino-4-methyl-coumarin-3-  
carboxamidomethyl)-cysteine.

## SEQ ID NO:5

A Q V D E V V D I M R V N V D K V L X R  
K L Z E L D D R A D A L Q A G A  
S

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and  
"Z" is S-(fluoresceinyl)-cysteine.

## SEQ ID NO:6

A Q V D E V V D I M R V N V D K V L E R K  
L dcC E L D D R A D A L Q A G A  
S

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and  
"dcC" is S-(7-dimethylamino-4-methyl-coumarin-3-  
carboxamidomethyl)-cysteine.

## SEQ ID NO:7

A Q V D E V V D I M R V N V D K V L E R K  
L mcp E L D D R A D A L Q A G A S

Where "X" is N(epsilon)-(2,4-dinitrophenyl)-lysine and  
"mcp" is 2-amino-3-(7-methoxycoumarin-4-yl)-propionic acid.

## SEQ ID NO:8

flG G G S N R T R I D E A N Q R A T R M L  
G G G C

Where flG is N-fluoresceinyl-glycine.

## SEQ ID NO:9

flG G G L S E L D D R A D A L Q A G A S Q  
F E T S A A K L K R K Y W W K N L K  
G G G C

Where flG is N-fluoresceinyl-glycine.

## SEQ ID NO:10

flG G G A Q V D E V V D I M R V N V D K V  
L E R D Q K L S E L D D R A D A L Q  
A G A S G G G C

Where flG is N-fluoresceinyl-glycine.

## SEQ ID NO:11

Z N K L K S S D A Y K K A W G N N Q D G  
V V A S Q P A R V V D E R E Q M A I

S G G F I R R V T N D A R E N E M D  
E N L E Q V S G I I G N L R H M A L  
D M G N E I D T Q N R Q I D R I M E  
K A D S N K T R I D E A N Q R  
A T K M L G S G C

Where Z is S-fluoresceinyl-cysteine.

SEQ ID NO:12

Z N K L K S S D A Y K K A W G N N Q D G  
V V A S Q P A R V V D E R E Q M A  
I S G G F I R R V T N D A R E N E  
M D E N L E Q V S G I I G N L R H  
M A L D M G N E I D T Q N R Q I D  
R I M E K A D S N K T R I D E A N  
Q A A T K M L G S G C

Where Z is S-fluoresceinyl-cysteine.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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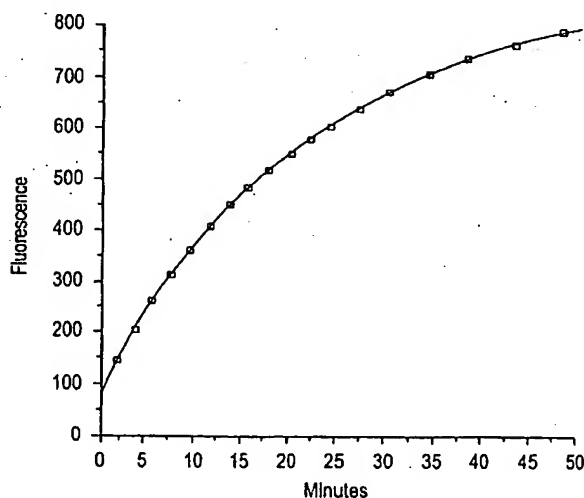
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18 September 2003

[Continued on next page]

(54) Title: HIGH-THROUGHPUT ASSAYS FOR THE PROTEOLYTIC ACTIVITIES OF CLOSTRIDIAL NEUROTOXINS



(57) Abstract: In this application is described substrates for high-throughput assays of clostridial neurotoxin proteolytic activities. Two types of substrates are described for use in assays for the proteolytic activities of clostridial neurotoxins: (1) modified peptides or proteins that can serve as FRET substrates and (2) modified peptides or proteins that can serve as immobilized substrates. In both types a fluorescent molecules is present in the substrate, eliminating the requirement for the addition of a fluorogenic reagent. The assays described can be readily adapted for use in automated or robotic systems.

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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/US 01/30188

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/542 C12Q1/37 G01N33/569 C07K7/08 C07K14/33

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE, FSTA, INSPEC, COMPENDEX, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 965 699 A (BOSTIAN KAREN A ET AL) 12 October 1999 (1999-10-12) cited in the application the whole document	1-3, 8-11,16, 17,49-54
A	WO 96 13607 A (PACKARD BEVERLY S; KOMORIYA AKIRA (US); ONCOIMMUNIN INC (US)) 9 May 1996 (1996-05-09) the whole document	1-3, 8-11,16, 17,49-54
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

19 February 2003

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

International Application No  
P., US 01/30188

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SOLEILHAC ET AL: "A sensitive and rapid fluorescence-based assay for determination of tetanus toxin peptidase activity" ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS, SAN DIEGO, CA, US, vol. 241, 1996, pages 120-127, XP002160418 ISSN: 0003-2697 cited in the application abstract	1-3, 8-11,16, 17,49-54
E	WO 01 92312 A (INST NAT SANTE RECH MED ;ROQUES BERNARD PIERRE (FR); ANNE CHRISTIN) 6 December 2001 (2001-12-06) the whole document	1,8,9,16
P,X	ANNE C ET AL: "HIGH-THROUGHPUT FLUOROGENIC ASSAY FOR DETERMINATION OF BOTULINUM TYPE B NEUROTOXIN PROTEASE ACTIVITY" ANALYTICAL BIOCHEMISTRY, ORLANDO, FL, US, vol. 291, no. 2, 15 April 2001 (2001-04-15), pages 253-261, XP001023970 ISSN: 0003-2697 the whole document	1,8,9,16
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# INTERNATIONAL SEARCH REPORT

ational application No.  
PCT/US 01/30188

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-3, 8-11, 16, 17, 49-54 partially

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-3,8-11,16,17,49-54 (partially)

A clostridial neurotoxin FRET substrate being a peptide according to SEQ ID NO : 1 and methods involving this substrate.

2. Claims: 1-3,8-11,16,17,49-54 (partially)

A clostridial neurotoxin FRET substrate being a peptide according to SEQ ID NO : 2 and methods involving this substrate.

3. Claims: 1,4,5,8,9,12,13,16,18,49-54 (partially)

A clostridial neurotoxin FRET substrate being a peptide according to SEQ ID NO : 3 and methods involving this substrate.

4. Claims: 1,4,5,8,9,12,13,16,18,49-54 (partially)

A clostridial neurotoxin FRET substrate being a peptide according to SEQ ID NO : 4 and methods involving this substrate.

5. Claims: 1,6-9,14-16,19,49-54 (partially)

A clostridial neurotoxin FRET substrate being a peptide according to SEQ ID NO : 5 and methods involving this substrate.

6. Claims: 1,6-9,14-16,19,49-54 (partially)

A clostridial neurotoxin FRET substrate being a peptide according to SEQ ID NO : 6 and methods involving this substrate.

7. Claims: 1,6-9,14-16,19,49-54 (partially)

A clostridial neurotoxin FRET substrate being a peptide according to SEQ ID NO : 7 and methods involving this substrate.

8. Claims: 20-22,29-32,39,40,44,45 (partially)

An immobilisable clostridium neurotoxin substrate being a

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

peptide according to SEQ ID NO : 8 and methods involving this substrate.

9. Claims: 20,29,30,39,44 (partially); 23,24,33,34,41, 46 (fully)

An immobilisable clostridium neurotoxin substrate being a peptide according to SEQ ID NO : 9 and methods involving this substrate.

10. Claims: 20,29,30,39,44 (partially); 25,26,35,36,42, 47 (fully)

An immobilisable clostridium neurotoxin substrate being a peptide according to SEQ ID NO : 10 and methods involving this substrate.

11. Claims: 20,27-30,37-39,42-44,47,48 (partially)

An immobilisable clostridium neurotoxin substrate being a peptide according to SEQ ID NO : 11 and methods involving this substrate.

12. Claims: 20,27-30,37-39,42-44,47,48 (partially)

An immobilisable clostridium neurotoxin substrate being a peptide according to SEQ ID NO : 12 and methods involving this substrate.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/US 01/30188

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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WO 9613607	A	09-05-1996	US 5605809 A	25-02-1997
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			WO 0192312 A1	06-12-2001



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